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ORIGINAL ARTICLE

Allozyme diversity levels in two congeneric *Dioon* spp. (Zamiaceae, Cycadales) with contrasting rarities

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Abstract Allozyme diversity and population genetic structure studies were conducted in populations of two Mexican cycad species occurring in adjacent and closely related biogeographic regions. We evaluated if rarity traits in *Dioon caputoi*, a micro-endemic species, and *Dioon merolae*, a regional endemic with a wider distribution, influence levels of genetic diversity in different ways. We also explored if genetic structure differs in these species, considering that they have similar population histories. Our results indicate that *D. caputoi* harbors lower levels of genetic diversity and allelic richness ($H_E = 0.358$, $P = 76.9$, $A_r = 1.86$) than *D. merolae* ($H_E = 0.446$, $P = 92.3$, $A_r = 2$). However, genetic structure does not differ between the two species despite their contrasting geographic distributions ($F_{ST} = 0.06$ vs. 0.07; *D. caputoi* and *D. merolae*, respectively). The comparison of population genetic structure information with historical and geographical aspects of the populations suggests that the rarity of *D. caputoi* might be due to relatively recent local ecological factors.

Keywords Allelic richness · Biogeographic history · *Dioon caputoi* · *Dioon merolae* · Genetic structure · Heterozygote excess · Zamiaceae

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Introduction

The concept of ecological rarity is still a polemical topic, although it is widely accepted that the main attributes that characterize rare species are small population sizes and narrow geographic distributions (Harper 1981; Rabinowitz 1981; Gaston 1994) relative to closely related species (Reveal 1981; Kruckeberg and Rabinowitz 1985). For over a decade, researchers have shown that in contrast to commonly distributed species, those with narrow geographic ranges tend to show low levels of genetic diversity (Loveless and Hamrick 1984; Karron 1987; Hamrick et al. 1991), a feature which may be related to higher probabilities of extinction (Lande 1988). These findings are in line with a natural expectation regarding genetic structures: while local populations should show higher levels of divergence due to drift and isolation, widespread species

will be composed of more tightly connected populations and will have less divergence, due to higher colonization abilities (Loveless and Hamrick 1984). On the other hand, Gitzendanner and Soltis (2000), Cole (2003), and Leimu et al. (2006) have indicated that this kind of comparison should take into account hypotheses of phylogenetic relationships. In population genetic studies that have considered the phylogenetic component, it has been observed—somewhat surprisingly—that similar genetic diversity levels and genetic structures (Morjan and Rieseberg 2004; Duminil et al. 2007) can coexist in congeneric or closely related species, regardless of their geographic range. In terms of genetic diversity levels, it is population size rather than geographic range that has the larger influence in the loss of population genetic diversity (Wright 1951; Ellstrand and Ellam 1993; Leimu et al. 2006), but the relative participation of these parameters is seldom analyzed clearly in rare versus common species comparisons (e.g., Maki et al. 2002; Ellis et al. 2006; Silva et al. 2007).

Previous studies in rare plant species have found that multiple factors related to their general biology and ecology (Hamrick et al. 1991; Duminil et al. 2007), as well as their population and evolutionary history (Kruckeberg and Rabinowitz 1985; Hewitt 1996; Aguinagalde et al. 2005) have a significant influence on the amount of genetic diversity and its organization among populations. This suggests that, in order to assess the influence of rarity per se (i.e., particularly population size) on levels of genetic diversity, factors such as reproductive system, phylogenetic relationships, and geographic history must be taken into account. Here, we conduct a population genetic study with two species of Mexican cycads in the genus *Dioon* that attempts to evaluate the interplay between rarity and the aforementioned factors in the shaping of genetic diversity among populations.

Cycads are a tropical and subtropical group of gymnosperms that are formally included in the order Cycadales (Norstog and Nicholls 1997; for a recent appraisal of phylogenetic relationships within the order, see Zgurski et al. 2008). They are dioecious, long-lived, and entomophilous (Norstog and Nicholls 1997); since their fossil history can be traced back nearly 300 million of years (Brenner et al. 2003), they probably constitute the most primitive group of seed plants alive. The genus *Dioon* Lindl. is one of the most common Neotropical genera and is endemic to Mexico—with the exception of the species *Dioon mejiae* Standl. & L.O. Williams, which is found in Honduras. On the Pacific Ocean side, the distribution of this genus spans a slope where the species *D. sonorense* (De Luca, Sabato & Vázq. Torres) Chemnick, T.J. Greg. & Salas M., Silvia, *D. tomasellii* De Luca, Sabato & Vázq. Torres, *D. holmgrenii* De Luca, Sabato & Vázq. Torres, and *D. stevensonii* Nicolalde-Morejón & Vovides can be found. On the other geographical side, the species *D. angustifolium* Miq. and *D. edule* Lindl. have a distribution that enters the Gulf of Mexico region, thereby constituting the wider geographic ranges within the genus. The remaining group of species is composed of *D. caputoi* de Luca, Sabato & Vázq. Torres, *D. argenteum* T.J. Greg. et al., *D. califanoi* De Luca & Sabato, *D. purpusii* Rose, *D. spinulosum* Dyer, *D. rezdowskii* De Luca et al., and *D. merolae* de Luca, Sabato & Vázq. Torres, all of which are located in central and southeastern Mexico, below the Trans-Mexican Volcanic Belt (Fig. 1). Besides its status as the most diverse biogeographic region for *Dioon* species, the latter area includes species with highly restricted geographic ranges, relative to those taxa distributed in northern Mexico. Within the central and southeastern Mexico group of *Dioon* species, *D. caputoi* and *D. merolae* have the most contrasting ranges (i.e., *D. caputoi* has the smallest and *D. merolae* the widest distribution areas).

Our choice of *D. caputoi* and *D. merolae* for the present population genetics study is directly related to their different geographic ranges, population sizes, and habitat specificity, features that might be related to the status of rarity. At the same time, it is important to note that these two taxa have important similarities in various aspects of their biology and natural history. First, the two species are closely related phylogenetically (González et al. 2008). Furthermore, since they lie in biogeographic regions with a close historical relationship (Contreras-Medina et al. 2007), there is a high probability that a common physiographic and climatic history has prevailed throughout their population history (Espinosa et al. 2002). In addition, their reproductive and dispersal systems might not have had a differential influence in their population dynamics given that in both *D. caputoi* and *D. merolae* these attributes are invariant—i.e., as is the case in all *Dioon* species, pollination is carried out by beetles and seeds are dispersed mainly by gravity (Norstog and Nicholls 1997). Homogeneity of biological, ecological, and historic factors is poorly considered in meta-analyses (cf. Gitzendanner and Soltis 2000; Cole 2003) and in focused population genetics case studies involving rare species (e.g., Mateu-Andrés and Segarra-Moragues 2000; Shapcott 2007; Silva et al. 2007).

Our choice of taxa in the present research might constitute an appropriate model to assess the consequences that different states of rarity might have for population genetic parameters in plant species. We specifically set out to compare genetic diversity levels and genetic population structures in a species with few, very small, and highly restricted populations, *Dioon caputoi*, with those of a species with larger and more widely distributed populations, *D. merolae*. We started with the expectation that genetic diversity would be lower in *D. caputoi* than in *D. merolae*. Our study has also considered that similarities

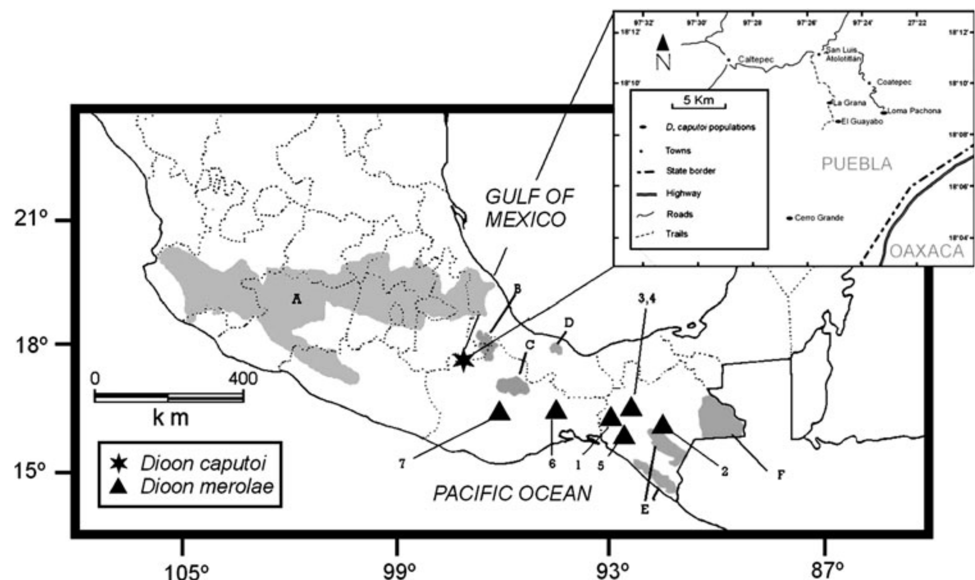

Fig. 1 Distribution of *Dioon caputoi* and *D. merolae*. *1* Las Minas, *2* Agua Prieta, *3, 4* Jiquipilas 1, 2, *5* Raymundo Flores, *6* Santiago Lachiguiri, *7* Loma Colorada. *A* Trans Mexican volcanic mountain range; *B–F* Floristic refugia: Córdoba, Sierra de Juárez, Los Tuxtlas, Soconusco, and Lacandonia (Cabrera-Toledo et al. 2008; Ferrusquía-Villafranca 1998; Toledo 1982)

in reproductive (i.e., pollen and seed-related) dispersal systems, as well as in evolutionary and biogeographic history, should determine a similar distribution of genetic variation within and between populations in each species, regardless of their present geographic range.

Materials and methods

Study area

Dioon caputoi and *D. merolae* are allopatric species, endemic to southern Mexico. They both grow in arid and semiarid environments, although the latter grows also in humid and sub-humid habitats. *D. caputoi* occurs only in the Tehuacán-Cuicatlán Biosphere Reserve, which is located in southern Puebla and northern Oaxaca within the sub-province Sierra Central de Oaxaca, which is part of the Sierra Madre del Sur (Ferrusquía-Villafranca 1998). This is a semi-arid region with high environmental heterogeneity and at least 29 vegetation types (Valiente-Banuet et al. 2000). The climate is characterized by summer rains and low temperature fluctuations, with an annual precipitation of 319.1 mm and a mean temperature of 18.1°C (García 2004). The *D. caputoi* habitat has a complex topography where flat areas and slight slopes are rare. The dominant vegetation is thorn scrub or Matorral Xerófilo (sensu Rzedowski 1978) with secondary succession elements (Valiente-Banuet et al. 2000).

Dioon merolae occurs in several localities of the Sierra Madre del Sur, in Oaxaca State and the Sierra Madre de Chiapas. The climate is warm humid and sub-humid in most of the localities, with summer rains and an annual precipitation of 1,018 mm and a mean temperature of 25.4°C (García 2004). However, there are some localities of *D. merolae* where we found precipitation from 1,500 to 3,000 mm and temperature from 10 to 15°C (Fortam 1984). In contrast to *D. caputoi*, which occurs only in dry scrub, populations of *D. merolae* are found also in tropical deciduous, tropical semi-deciduous, and pine-oak forests.

Species studied

Dioon caputoi and *D. merolae* are two long-lived, entomophilous, dioecious, and arborescent cycads. Individuals of *D. caputoi* have erect or prostrate trunks, up to 2 m in height and 20–25 cm in diameter. This cycad species is one of the most geographically restricted of the genus and is composed of very small populations. Only four populations of the species have been recorded, unique to a small area of the Mexican state of Puebla (Fig. 1), and they are comprised of 60–100 adults each; the greatest distance between any of these populations is 9.4 km (i.e., Loma Pachona and Cerro Grande, Fig. 1).

Plants of *D. merolae* are larger, reaching 5 m in height and 70 cm in diameter. The species is regionally endemic to southeastern Mexico (Fig. 1), is allopatric to *D. caputoi*, and has a much wider distribution. So far, *D. merolae* has been reported in seven localities in the Mexican state of Chiapas and at least four in Oaxaca (Moretti et al. 1993). Population sizes range from 100 to 1,000 adult plants, and 440 km separate the most distant populations (i.e., Agua Prieta and Loma Colorada, Fig. 1).

According to the International Union for Conservation of Nature (IUCN), the conservation statuses of *D. caputoi* and *D. merolae* are "critically endangered" and "vulnerable," respectively (IUCN 2009).

Sample collection

We used population genetic data from our previous study on *D. caputoi* (see Cabrera-Toledo et al. 2008). Briefly, we collected samples, from 30 to 50 individuals per population, throughout the complete geographic range of the four known populations, namely La Grana, El Guayabo, Loma Pachona, and Cerro Grande (Fig. 1). Additionally, we collected *D. merolae* materials, consisting of a random sample of leaflets of 30 adult plants from seven populations distributed in approximately 3 ha plots per population, covering most of the geographical range of the species (ca. 70%; Table 1). The collected leaflets were transported on ice to the laboratory to avoid protein denaturalization and were stored at −70°C until protein extraction.

Enzyme extraction and electrophoresis

Approximately 250 mg of leaflet tissue per *Dioon merolae* plant was ground in liquid nitrogen and about 250 µl of extraction buffer (0.1 M Tris-HCl pH 7.5, 4% PVP-40, 0.001 M EDTA, 0.01 M $CaCl_2$, 0.01 M $MgCl_2$ and 0.1% β-mercaptoethanol; González-Astorga et al. 2003) were added to dilute and stabilize the enzyme extracts. These extracts were then stored on filter paper wicks at −70°C until subsequent use. Multilocus genotypes from the sampled individuals were obtained through horizontal starch gel electrophoresis (12% w/v). Electrophoresis was carried out in two buffer systems: R (Li et al. 1999) and PK (Yang and Meerow 1996). For system R, electrode buffer (pH 8.0) consisted of 0.4 M Tris, 0.105 M citric acid monohydrate;

Table 1 Allozyme systems [nomenclature and abbreviations follow Wendel and Weeden 1989, based on IUBNC Enzyme Commission number (E.C.no.)] and allele frequencies of 13 allozyme loci in seven populations of *Dioon merolae*: *1* Las Minas, *2* Agua Prieta, *3* Jiquipilas 1, *4* Jiquipilas 2, *5* Raymundo Flores, *6* Santiago Lachiguiri, and *7* Loma Colorada

Allozyme/E.C.no./buffer system	Loci	Alleles	Population						
			1	2	3	4	5	6	7
Anodic Peroxidase/1.11.1.7/R	*APX1*	1	0.575*	0.672	0.696	0.707*	0.533*	0.750	0.667
		2	0.425*	0.328	0.304	0.293*	0.467*	0.250	0.333
	APX2	1	0.500*	0.309*	0.407*	0.481*	0.500*	0.452*	0.342*
		2	0.500*	0.691*	0.593*	0.519*	0.500*	0.548*	0.658*
	APX3	1	0.500*	0.485*	0.438*	0.500*	0.500*	0.375*	0.520*
		2	0.500*	0.515*	0.563*	0.500*	0.500*	0.625*	0.480*
	APX4	1	1.000	1.000	1.000	1.000	1.000	0.967	0.520*
		2	0.000	0.000	0.000	0.000	0.000	0.033	0.480*
Malate-dehydrogenase/1.1.1.37/R	*MDH1*	1	0.500*	0.441*	0.344*	0.367*	0.339*	0.444	0.190
		2	0.500*	0.559*	0.656*	0.633*	0.661*	0.556	0.810
	MDH2	1	0.591*	0.353*	0.563	0.500*	0.500	0.536	0.450
		2	0.409*	0.647*	0.438	0.500*	0.500	0.464	0.550
Isocitrate dehydrogenase/1.1.1.41/R	*IDH*	1	0.406*	0.515*	0.219*	0.190	0.383*	0.054	0.207*
		2	0.188*	0.132*	0.281*	0.293	0.150*	0.429	0.500*
		3	0.406*	0.353*	0.500*	0.517	0.467*	0.518	0.293*
6-Phosphogluconate dehydrogenase/5.3.1.9/R	*6PGD*	1	0.455*	0.485*	0.397*	0.583*	0.241	0.500*	0.442*
		2	0.545*	0.515*	0.603*	0.417*	0.759	0.500*	0.558*
Glutamate oxaloacetate transaminase/2.6.1.1/PK	*GOT*	1	0.580*	0.426*	0.125	0.050	0.500*	1.000	0.383*
		2	0.420*	0.574*	0.813	0.900	0.500*	0.000	0.617*
		3	0.000*	0.000*	0.063	0.050	0.000*	0.000	0.000*
Diaphorase/1.6.99/PK	*DIA*	1	0.500*	0.485*	0.594	0.500*	0.417*	0.500*	0.482*
		2	0.500*	0.515*	0.406	0.500*	0.583*	0.500*	0.518*
Phosphogluco isomerase/5.3.1.9/R	*PGI*	1	0.727*	0.500*	0.276*	0.554*	0.466*	0.300	0.417*
		2	0.273*	0.500*	0.724*	0.446*	0.534*	0.700	0.583*
Esterase/3.1.1/R	*EST*	1	0.318*	0.500*	0.422	0.567*	0.519*	0.655*	0.586*
		2	0.682*	0.500*	0.578	0.433*	0.481*	0.345*	0.414*
Menadione reductase/1.6.99/R	*MNR*	1	0.500*	0.500*	0.500*	0.500*	0.500*	0.404*	0.450*
		2	0.500*	0.500*	0.500*	0.500*	0.500*	0.596*	0.550*

* $p < 0.01$; statistical significance from Hardy–Weinberg deviations, using the conventional Monte Carlo method (10 batches, 10,000 permutations per batch, and 100,000 total permutations)

gel buffer (pH 8.0) consisted of 0.009 M Tris, 0.005 M histidine-HCl. This system was carried out at 4°C for 8.5 h (constant current of 35 mA and voltage of 200 V). For system PK, electrode buffer (pH 8.1) consisted of 0.3 M H_3Bo_3, 0.056 M NaOH; gel buffer PK (pH 8.7) consisted of 0.083 M Tris, 0.005 M citric acid monohydrate. This system was carried out at 4°C for 7 h (constant current of 50 mA and voltage of 200 V). A total of nine allozyme systems were selected from Cabrera-Toledo et al. (2008), which were the ones that showed clear staining in both species. Enzyme names and buffer systems are shown in Table 1. In each activity zone we recorded banding patterns as the basis for the genetic estimators.

Genetic variation analysis

We estimated allele frequencies for each of the allozyme systems indicated in Table 1. For *D. caputoi*, we used data from Cabrera-Toledo et al. (2008); for *D. merolae*, we selected a final set of 13 loci (the same obtained in this study for *D. caputoi*, Table 1). We used the software program TFPGA 1.3 (Miller 1997) to calculate the following four population genetic parameters: the observed mean heterozygosity (H_O), the mean number of alleles per locus (A), the percentage of polymorphic loci at the 95% probability criterion (P), and the expected mean heterozygosity (H_E) under Hardy–Weinberg equilibrium (Hedrick 2000). Since allele diversity is sensitive to the presence or absence of rare alleles (Kalinowski 2004), we also calculated a parameter of allelic richness (A_r), using a rarefaction technique in order to deal with the sample size effect in this parameter (Kalinowski 2005). Exact tests were used to test for deviations from the expected genotypic frequencies under Hardy–Weinberg equilibrium. Genetic variation parameters were compared between species using the nonparametric Mann–Whitney U test as implemented in StatSoft (2002).

Genetic structure analysis

In order to identify the proportion of total genetic variation occurring within and among populations, an analysis of molecular variance (AMOVA; Excoffier et al. 1992) and pairwise F_{ST} comparisons based on AMOVA were performed in both species by using GenAlex 6 (Peakall and Smouse 2006). The significance of the variance components and Φ_{ST} statistics as well as pairwise comparisons were tested using a permutational approach, eliminating the normality assumption that is conventional for analysis of variance, but inappropriate for molecular data (Excoffier et al. 1992). In order to test for isolation by distance (IBD), we calculated log geographic distance and pairwise genetic differentiation values $[F_{ST}/(1 - F_{ST})]$ as suggested by Rousset (1997). Then, we used these values to perform a Mantel test between populations in both species using TFPGA 1.3 (Miller 1997).

Inbreeding coefficients were calculated using TFPGA 1.3. In order to determine whether F_{IS} and F_{IT} estimates for each locus were significantly different from zero, X^2 tests $[X^2 = F\ (2N)\ (k - 1)]$ were performed using $k\ (k - 1)/2$ degrees of freedom, where N is the sample size, and k the number of alleles (Weir 1990). The 95% CI of the inbreeding coefficients was obtained by two re-sampling methods, bootstrap over loci for the multi-locus estimate and jackknife over populations for the single-locus estimates (Weir and Cockerham 1984). Inbreeding coefficients as well as pairwise comparisons (F_{ST}) parameters were compared between species using the nonparametric Mann–Whitney U test (StatSoft 2002). Finally, phenetic clustering of populations within species was determined using Nei's (1972) genetic distances and the unweighted pair group method with arithmetic mean (UPGMA) (Sneath and Sokal 1973) algorithm, as implemented in TFPGA 1.3 (Miller 1997).

Results

Genetic variation

We obtained data for 13 loci from the nine allozyme systems used. The allele frequencies of these loci are shown in Table 1 for the seven populations analyzed in *Dioon merolae* (for *D. caputoi*, see Table 1 in Cabrera-Toledo et al. 2008). All loci were polymorphic in at least one population of both species. Numbers of alleles per locus (A) averaged 1.98 and 2.00 for *D. caputoi* and *D. merolae*, respectively; the difference between these two values was not significant (Table 2, $p = 0.13$ in Mann–Whitney test). In contrast, allelic richness (A_r) was significantly lower in *D. caputoi* (Table 2; $p < 0.01$ Mann–Whitney test) than in *D. merolae*. Differences were also significant (Table 2; $p < 0.01$ in Mann–Whitney test) in comparisons between *D. caputoi* and *D. merolae* for the following parameters: mean observed heterozygosity (H_O; 0.522 vs. 0.713, respectively), mean expected heterozygosity (H_E; 0.358 vs. 0.446, respectively), and mean percentage of polymorphic loci (P; 76.9 and 92.3, respectively). Additionally, a mean of six loci tended towards heterozygote excess in *D. caputoi* with respect to Hardy–Weinberg equilibrium (ca. 60% of polymorphic loci), whereas in *D. merolae* the same occurred for a mean of nine loci (ca. 70% of polymorphic loci). Given that nine enzymes were selected from Cabrera-Toledo et al. (2008), genetic diversity values in this study are slightly different from those reported previously for *D. caputoi*.

Table 2 Genetic variation parameters for 13 allozyme loci in *Dioon caputoi* and *D. merolae*

Species	Population	*N*	*A*	A_r*	H_O*	H_E*	P-95*
Dioon caputoi	1. La Grana	30.4	2.0	1.83	0.530	0.358	69.2
	2. El Guayabo	49.5	2.0	1.91	0.466	0.340	84.6
	3. Loma Pachona	48.5	2.0	1.89	0.530	0.374	76.9
	4. Cerro Grande	53.8	1.92	1.80	0.563	0.361	76.9
Mean ± SD		45.5 ± 19.36	1.98 ± 0.04	1.86 ± 0.04	0.522 ± 0.04	0.358 ± 0.014	76.9 ± 6.29
Dioon merolae	1. La Minas	30.2	2.0	2.0	0.779	0.455	92.3
	2. Agua Prieta	33.2	2.0	1.99	0.767	0.454	92.3
	3. Jiquipilas 1	30.8	2.1	2.03	0.638	0.433	92.3
	4. Jiquipilas 2	29.1	2.1	1.99	0.730	0.434	92.3
	5. Raymundo Flores	29.3	2.0	1.99	0.754	0.454	92.3
	6. Santiago Lachiguiri	26.6	2.0	1.92	0.606	0.407	84.6
	7. Loma Colorada	27.2	2.1	2.07	0.721	0.481	100
Mean ± SD		29.5 ± 2.23	2.0 ± 0.053	2 ± 0.02	0.713 ± 0.066	0.446 ± 0.023	92.3 ± 4.45

N Mean number of plants evaluated, i.e., the sample that is finally computed, since missing genotypes are captured as zero, *A* average number of alleles per locus, A_r allelic richness, H_O, H_E observed and expected heterozygosity, respectively, *P-95* percentage of polymorphic loci at 95% criterion

* $p < 0.05$; significant differences (Mann–Whitney test) between species

Genetic structure

The AMOVA test (Table 3) showed a similar distribution of genetic variation for both species, with a higher percentage occurring within populations (83% in *D. caputoi* vs. 80% in *D. merolae*). For both species, the global genetic differentiation between populations was significant (for *D. caputoi*, $\Phi_{ST} = 0.169$ and $p < 0.05$; for *D. merolae*, $\Phi_{ST} = 0.195$ and $p < 0.05$). The mean values of pairwise F_{ST} comparisons did not differ significantly between species ($p = 0.41$ in Mann–Whitney test). In *D. caputoi*, the F_{ST} values were found to differ from zero ($F_{ST} = 0.06 \pm 0.02$; $p < 0.05$) in all population pairs, whereas in *D. merolae* ($F_{ST} = 0.07 \pm 0.03$) one comparison did not differ significantly from zero (Agua Prieta vs. Raymundo Flores, $p = 0.07$). The correlation between pairwise $[F_{ST}/(1 - F_{ST})]$ and log geographic distance was significant only for *D. merolae* ($r = 0.547$, $p < 0.05$).

Mean values of inbreeding coefficients for *D. caputoi* and *D. merolae*, respectively, were as follows: $F_{IT} = -0.354$ and -0.484 and $F_{IS} = -0.452$ and -0.592. All inbreeding coefficients were significantly negative ($p < 0.05$; Table 4), indicating a global (F_{IT}) and local (F_{IS}) heterozygote excess for each species. These excesses were higher for *D. merolae* ($p < 0.001$ in Mann–Whitney test). Finally, the UPGMA tree based on Nei's (1972) genetic distances revealed two major groups (i.e., *D. caputoi* and *D. merolae*; Fig. 2; Table 5).

Discussion

Genetic variation

Both species have high levels of genetic diversity (as measured by H_O, H_E, *P*, and A_r) but *Dioon caputoi* shows

Table 3 Analysis of molecular variance (AMOVA) for 185 individuals from four populations of *Dioon caputoi* and 219 individuals from seven populations of *D. merolae*

Source	Summary AMOVA									
	Dioon caputoi					*Dioon merolae*				
	df	SS	MS	Est. var.	Percentage	*df*	SS	MS	Est. var.	Percentage
Among pops	3	96.890	32.297	0.638	17	6	212.027	35.338	0.999	20
Within pops	181	568.207	3.139	3.139	83	212	871.672	4.112	4.112	80
Total	184	665.097		3.778	100	218	1,083.699		5.110	100
Φ_{ST}	0.169 (p = 0.010)						0.195 (p = 0.010)			

p Probability value (rand ≥ data)

Table 4 Inbreeding coefficients for 13 polymorphic loci in four populations of *Dioon caputoi* and seven of *D. merolae*

Locus	*Dioon caputoi*		*Dioon merolae*	
	F_{IT}	F_{IS}	F_{IT}	F_{IS}
APX1	−0.389*	−0.472*	−0.493*	−0.609*
APX2	−0.356*	−0.451*	−0.463*	−0.576*
APX3	−0.354*	−0.449*	−0.450*	−0.564*
APX4	−0.378*	−0.476*	−0.499*	−0.595*
MDH1	−0.305*	−0.408*	−0.490*	−0.603*
MDH2	−0.338*	−0.437*	−0.507*	−0.624*
IDH	−0.374*	−0.455*	−0.517*	−0.627*
6PGD	−0.290*	−0.392*	−0.469*	−0.579*
GOT	−0.355*	−0.450*	−0.532*	−0.598*
DIA	−0.325*	−0.429*	−0.462*	−0.577*
PGI	−0.400*	−0.499*	−0.494*	−0.599*
EST	−0.401*	−0.475*	−0.475*	−0.586*
MNR	−0.327*	−0.432*	−0.446*	−0.560*
Mean	−0.354a	−0.452c	−0.482b	−0.592d
SD	0.117	0.098	0.087	0.070
95% Confidence interval	−0.138 to −0.570	−0.256 to −0.620	−0.326 to −0.648	−0.468 to −0.718

* $p < 0.05$; significance (X^2 tests), i.e. heterozygote excess, is present; *different letters* indicate differences between species parameters

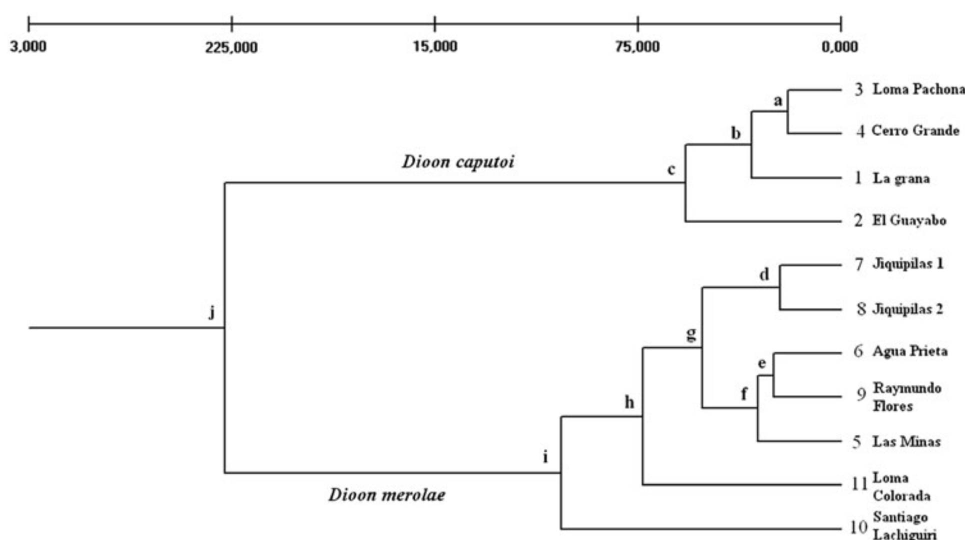

Fig. 2 Unweighted pair group method with arithmetic mean phenogram based on Nei's (1972) genetic distances among 11 populations of two species of *Dioon*, estimated from 13 loci (Table 5)

the lower values. Our results agree with those reported by Hamrick et al. (1991) and more recently by Leimu et al. (2006). Using a comparative meta-analysis for 12 angiosperm species, these authors found less genetic diversity in species with small population sizes. Our previous, directly relevant study on *D. caputoi* (Cabrera-Toledo et al. 2008) showed that the mean population values of genetic diversity (i.e., % *P*, H_O, and H_E) for this species do not differ significantly from those of the other congeneric species studied so far—*D. edule, D. angustifolium, D. sonorense*, and *D. tomasellii*—even though these other taxa have different population sizes and geographic ranges. As was mentioned in the "Introduction," reproductive and dispersal systems, which are relevant in principle for the

Table 5 Nodes of genetic distances of Nei's (1972) in 11 populations of two species of *Dioon* in central and southeastern Mexico (Fig. 2)

Node	Genetic distances	Includes populations
a	0.02	3, 4
b	0.03	1, 3, 4
c	0.06	1, 2, 3, 4
d	0.02	7, 8
e	0.02	6, 9
f	0.03	5, 6, 9
g	0.05	5, 6, 7, 8, 9
h	0.07	5, 6, 7, 8, 9, 11
i	0.1	5, 6, 7, 8, 9, 10, 11
j	0.23	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11

shaping of genetic diversity and structure (Hamrick et al. 1991), are invariant in this cycad genus (Norstog and Nicholls 1997). By virtue of their status as outcrossing, long-lived plants, it was expected that *Dioon* species would show similar levels of genetic diversity; however, genetic diversity of *D. caputoi* populations did differ from that present in *D. merolae*. As both species are closely related, both in phylogenetic (González et al. 2008) and in biogeographic terms (Morrone et al. 1999; Contreras-Medina et al. 2007), it is highly likely that a common evolutionary, physiographic, and climatic history has influenced their population dynamics in similar ways. Hence, a comparison with respect to rarity per se could be more appropriate to show the effect of small populations on genetic diversity.

Genetic structure

As expected, the highest proportion of overall genetic variation was found within populations in both species, with just over one-quarter of it distributed among populations (Table 3). This pattern is in agreement with the findings of Hamrick and Godt (1996) and Hamrick (2004) for other long-lived tree species. Likewise, the genetic structure estimated from pairwise F_{ST} values did not differ between both species, despite the mean geographic distance between pairs of populations being significantly smaller in *D. caputoi* than in *D. merolae* (5.46 ± 3.2 vs. 168.77 km ± 115; $F = 11.76$, $p = 0.002$). Both species have F_{ST} values comparable with other congeners that have wider distributions, for example, *D. edule* ($F_{ST} = 0.075$, González-Astorga et al. 2003) and *D. holmgrenii* ($F_{ST} = 0.069$, González-Astorga et al. 2008). These two species have northern geographic ranges in the vicinity of 950 and 100 km, respectively, which are more comparable to the ca. 400 km that divides the two most distant populations of *D. merolae*. In contrast, in the case of *D. caputoi*, only ca. 10 km separates the most distant populations. It is important to recall that population structure is also similar between *D. merolae* and *D. caputoi* (with adult plants being the most representative ones in populations), and that recruitment rate is poor, especially in *D. caputoi* (Lázaro-Zermeño 2002; Flores-Vázquez 2008; Cabrera-Toledo 2009); it is therefore likely that we are comparing ancient contemporary populations. If that is the case, then similar genetic structures in species with such different actual geographic ranges are probably explained by the closely related evolutionary and biogeographic history. Aguinagalde et al. (2005) have suggested that aspects related to the geographical and historical context in which species are evolving—such as the size or location of the distribution range—rather than life history traits, explain the actual population differentiation in European long-lived trees with boreal-temperate and temperate distribution. This seems to be the case also for the *D. caputoi* and *D. merolae* species pair. In contrast, for herbaceous plants, other biological traits have had more influence in the shaping of genetic structure (Moyle 2006).

Results of the Mantel test indicate that isolation by distance has occurred in *D. merolae* but not in *D. caputoi*. This means that genetic structure in *D. merolae* might be driven mainly by gene flow (Slatkin 1985). On the other hand, genetic drift is probably the predominant process underlying genetic structure in *D. caputoi* (cf. Knowles et al. 2007). In this context, it could be hypothesized that *D. caputoi* used to have a wider geographic range (perhaps similar to *D. merolae*) and has recently experienced contraction. Since genetic diversity is distributed mostly within populations rather than among populations, this hypothetical situation might not have substantially affected genetic diversity at the species level. An alternative explanation is that our collected data represent only a proportion of the geographic range. There is preliminary information available on the existence of at least two additional populations of *D. caputoi* (J. M. Salazar, pers. comm.) in Oaxaca. Although their taxonomic status has not been confirmed yet by specialists, these populations might constitute valuable sources of additional evidence to evaluate the latter scenario.

With respect to heterozygote excess, hypothetical explanations have been postulated previously for *Dioon*. These have included balancing selection (González-Astorga et al. 2003) and drift. The latter process might have been the consequence of dissimilar allele frequencies between male and female plants (Cabrera-Toledo et al. 2008). Evidence found in the present study does not support the balancing selection hypothesis, since several loci in both species presented heterozygote excess, i.e., there is a low probability that the same selection process has simultaneously shaped variation in all loci. According to the climatic history context discussed above, and considering the presence of Pleistocene refugia (Toledo 1982) surrounding the distribution of both species (Fig. 1), here we propose a third hypothetical explanation. Hewitt (1996) suggested that climate change dynamics affecting Pleistocene refugia (warm interglacial and temperate glacial periods) probably generated processes of expansion and contraction that were not so drastic as to cause extinction, but strong enough to produce considerable genome reorganization during each climate change interval. This may have been particularly true for southern and southeastern Mexico, where extensive environmental fluctuation affecting habitats apparently took place over at least 4 million years (Graham 1998). We therefore postulate that the heterozygote excess found in the two species studied is a consequence of this long-lasting process. The steps that we envision for these climate-related events can be ordered

in the following way. First, allelic frequencies may have changed differentially, as a consequence of periods of contraction during the Ice Ages (cf. Hewitt 2000). Later expansion and re-colonization by founders, differing in allelic composition and originating from distinct areas (during the interglacials), may have promoted high heterozygote frequencies in the populations (Milkman 1975; Hewitt 1996; Comps et al. 2001). Finally, a recolonization process might have then taken place. Support for this last explanation comes from the IBD pattern found in *D. merolae* but not in *D. caputoi* data. A reasonable ad hoc explanation for this exception might be related to the latter's lower recruitment capacity (Cabrera-Toledo 2009).

Further phylogeographic research with DNA molecular markers would be necessary to corroborate the hypothetical model of the causes of heterozygote excess in *Dioon* presented here, as well as other questions regarding historical demography (sensu Avise 2009), e.g., which evolutionary coalescence times are characteristic of each genomic compartment. Recently, as part of an ongoing project on cycad DNA barcoding, our research group calculated the number of DNA diagnostic characters present in all species of *Dioon* for a suite of chloroplast coding (namely, *mat*K, *rpo*B, *rpo*C1, and *rbc*L) and noncoding regions (*trn*H-*psb*A, *atp*F-*atp*H, and *psb*K-*psb*I; Nicolalde-Morejón et al. 2010). In that study, we found that the intergenic spacer *psb*K/I displays the largest number of DNA diagnostics for both *Dioon caputoi* (22 diagnostic sites out of 680 aligned nucleotides) and *D. merolae* (7 diagnostics out of 680 sites), as well as for the remaining *Dioon* species. A more general finding, however, was that neither the two additional noncoding chloroplast loci (*trn*H-*psb*A and *atp*F-*atp*H) nor the coding regions that were successfully amplified in *Dioon* (*rpo*B, *rpo*C1, and *rbc*L) showed any significant nucleotidic variation.

Although it is evident that our work is not phylogeographically oriented, it is possible to draw preliminary comparisons between the patterns of variation in our molecular *Dioon* datasets and phylogeographic inferences derived from DNA data in other cycad genera. For instance, in a study of the differential evolutionary histories of Japanese populations of *Cycas revoluta* and *C. taitungensis*, Chiang et al. (2009) noticed high levels of variation in the *atp*B-*rbc*L intergenic spacer, which is in turn interpreted by the authors as an indication of relatively ancient origins of these populations, after which mutations steadily accumulated. It might be premature to assume that species in other cycad genera have overall contrasting patterns of molecular evolution with respect to *Dioon*; instead, the aforementioned work in *Cycas* (see also Huang et al. 2001; Kyoda and Setoguchi 2010) encourages us to evaluate additional regions in other genomic compartments, at the population level, in order to infer phylogeographic history in *D. caputoi*, *D. merolae*, and other species in the genus. In line with these future aims, we finally add that in a recent test of approximately 100 ISSR primers we have already found 14 and 19 polymorphic markers in *D. caputoi* and *D. merolae*, respectively (González-Astorga et al., unpublished observations). ISSR variation has also been recently examined for *Cycas* species from a phylogeographic and conservation genetics perspective (e.g., Xiao et al. 2004, 2005; Jianguang et al. 2005; Xiao and Gong 2006); we consider that analysis of these markers might also contribute to deepening our knowledge of the spatiotemporal aspects of genetic diversity in *Dioon* species.

Conclusions

Our findings in the present study provide additional support for earlier characterizations of *Dioon* as a genus with high levels of genetic diversity. As reported in previous studies, genetic diversity in *D. caputoi* is similar to that in congeneric species with contrasting biogeographic histories, even though its population sizes and distribution ranges are limited, and it shows greater habitat specificity. However, the present study demonstrates that, when compared to the biogeographically close species *D. merolae*, *D. caputoi* shows decreased levels of genetic diversity. This evidence further suggests that when the population genetic condition of a rare species is evaluated, it is crucial to take into account not only life history-related, but also historical (i.e., evolutionary and biogeographic) aspects that might be relevant in the shaping of genetic structure. In this particular study, the role of past population history is indicated by the fact that both share a similar organization of genetic diversity, in spite of their divergent distribution ranges.

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